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DOI: https://doi.org/10.1016/B978-0-12-385928-0.00010-9

Glycoprotein maturation and the UPR

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Running title: N- and O-glycosylation analysis

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ABSTRACT

Glycosylation is a complex form of protein modification occurring in the secretory pathway. The addition of N- and O-glycans affects intracellular processes like the folding and trafficking of most glycoproteins. To better understand the impact of glycosylation in protein folding and maturation, parameters like glycosylation site occupancy and oligosaccharide structure must be measured quantitatively. In the present chapter, we describe current methods enabling the determination of N-glycosylation by assessment of cellular dolichol-phosphate levels, dolichol-linked oligosaccharides and the occupancy of N-glycosylation sites. We also provide detailed methods for the analysis of O-glycosylation, whose role in intracellular protein maturation is often overlooked.

1. INTRODUCTION

Glycosylation is a widespread and complex form of modification, which adds signals and specific functions to glycoproteins. N-linked and O-linked glycosylation, which are the main forms of protein glycosylation, are structurally and functionally distinct. N-linked glycosylation is initiated in the endoplasmic reticulum (ER), where the oligosaccharide $GlcNAc_2Man_9Glc_3$ is transferred co-translationally to selected asparagine residues of nascent glycoproteins. While still in the ER, oligosaccharides on glycoproteins are trimmed by glucosidase and mannosidase enzymes to $GlcNAc_2Man_8$. Later in the Golgi apparatus N-linked oligosaccharides undergo further trimming and elongation steps, which contribute to the structural diversity of N-linked glycosylation. In contrast to the co-translational beginning of N-glycosylation, O-linked glycosylation takes place on folded proteins and is initiated by the transfer of monosaccharides to serine and threonine residues. Some forms of O-glycosylation like O-fucosylation (Harris and

Spellman, 1993) and O-mannosylation (Lommel and Strahl, 2009) begin in the ER, whereas mucin-type O-glycosylation (Tian and Ten Hagen, 2009) and the biosynthesis of glycosaminoglycan chains (Bishop et al., 2007) begin in the Golgi apparatus.

N-glycosylation is important for the folding and trafficking of many glycoproteins (Helenius and Aebi, 2004). The inhibition of N-linked glycosylation, for example by tunicamycin, results in the accumulation of misfolded proteins in the ER, which is a strong stimulus of the UPR (Shamu et al., 1994). After trimming by glucosidase-I in the ER, N-linked oligosaccharides are bound by the chaperone proteins calnexin and calreticulin, which contribute to the folding of glycoproteins (Ellgaard et al., 1999). Furthermore, N-linked oligosaccharides are also recognized by the EDEM protein, which redirects misfolded proteins from the ER lumen to the cytosol for proteasome-mediated degradation. Another important signal carried by N-linked oligosaccharides is the mannose-6-phosphate epitope, which mediates the recognition and transfer of lysosomal proteins to their target organelle (Dahms et al., 1989).

The most common form of O-glycosylation, i.e. mucin-type glycosylation, does not contribute to glycoprotein folding since it occurs in the Golgi apparatus. However, the trafficking and secretion of some glycoproteins requires proper mucin-type glycosylation, as demonstrated in the case of the hormone FGF23. Deficient initiation of mucin-type O-glycosylation alters the susceptibility of FGF23 to Golgi-localized proteases, thereby inhibiting the maturation of active FGF23. Mucin-type glycosylation of FGF23 is initiated by the polypeptide N-acetylgalactosaminyltransferase-3 enzyme. Loss of this enzymatic activity leads to the disease tumoral calcinosis, which is also caused by FGF23 deficiency (Chefetz and Sprecher, 2009).

Another example of glycosyltransferase-assisted trafficking is given by the OFUT1 O-fucosyltransferase enzyme. This ER-localized glycosyltransferase adds fucose to the

EGF-like repeats of proteins such as Notch and its ligands. Independent from its glycosyltransferase activity, OFUT1 also acts as a chaperone, which is essential for the transfer of Notch from the ER to the Golgi apparatus (Okajima et al., 2005). The contribution of O-linked glycosylation to glycoprotein folding and secretion is much less documented than in the case of N-linked glycosylation. This limited knowledge is certainly related to the technically difficult investigation of O-linked glycan structures. In fact, O-linked glycans are often densely clustered and these glycans cannot be released without significantly altering the polypeptide backbone. The O-glycanase enzyme from *Streptococcus pneumoniae* has a specificity limited to the O-linked disaccharide Gal(β1-3)GalNAc-O. By contrast, N-linked glycans can be conveniently cleaved at the first GlcNAc unit by the N-glycosidase F.

2. N-GLYCOSYLATION

Proper N-linked glycosylation is required for folding and intracellular trafficking of glycoproteins. Deficiency in the biosynthesis of the precursor of N-glycans or in the processing of N-glycans in the ER can alter glycoprotein folding and induce UPR. The following methods can be applied to identify defects of oligosaccharide assembly and of N-glycosylation site occupancy in target cells.

2.1. Dolichol phosphate analysis

The oligosaccharide core consisting of $GlcNAc_2Man_9Glc_3$ is first assembled on the polyisoprenoid carrier dolichol (Dol). Moreover, dolichol-phosphate (Dol-P) is also part of the glycosylation substrates Dol-P-Man and Dol-P-Glc, which account for the final

extension of the oligosaccharide core in the ER. Considering the low levels of Dol-P based structures in cells, a sensitive Dol-P detection can be achieved by labeling with the fluorescent compound 9-anthryldiazomethane. The procedure was originally described for tissue sample extraction (Elmberger et al., 1989) and we have adapted the method for cultured cells (Haeuptle et al., 2009). Detection of unlabeled Dol-P can also be achieved by negative ion electrospray mass spectrometry (ESI-MS), which, in combination to precursor ion fragmentation, provides detailed information on Dol-P composition and on the saturation state of the α-isoprene unit.

2.1.1. Required materials

Devices and materials

- LaChrom D-7000 HPLC system (Merck), equipped with an Inertsil ODS-3 column (5 µm, 4.6 x 250 mm; GL Sciences Inc., Japan) with a precolumn and a LaChrom 7485 fluorescence detector.
- Nano-flow ESI-ion trap MS (Eksigent nano-LC, 3200 QTRAP-MS, AB/SCIEX)
- C_{18} Sep-Pak and Silica Sep-Pak cartridges (Waters)

Other reagents

- 9-anthryldiazomethane (ADAM) (Sigma-Aldrich)
- C_{80}-polyprenol and C_{95}-Dol-P standards (Larodan Fine Chemicals, Sweden)
- Diazald (Sigma-Aldrich)
- Carbitol (Sigma-Aldrich)

2.1.2. Extraction and purification of Dol and Dol-P

Adherent cells (about 3 x 10^8 cells) are washed in PBS after trypsinization and resuspended in 6 ml of water and then fixed by addition of 6 ml of methanol. The resulting suspension is transferred to a round bottom glass flask and 3 ml of 15 M potassium hydroxide are added for alkaline hydrolysis. An aliquot of the C_{80} polyprenyl-phosphate (e.g. 15 µg) can be added as internal standard for subsequent quantification. The round bottom flask is attached to a reflux cooler and incubated in a preheated oil bath at 100 °C for 1 h. This alkaline hydrolysis removes carbohydrates from the Dol-P carrier. The resulting lysate is transferred to a glass tube and methanol is added to a total volume of 15 ml. The suspension is mixed with 30 ml of methanol/dichloromethane (1:4, v:v) and incubated at 40 °C for 1 h. Discard the upper phase (methanol) and transfer the lower phase (dichloromethane) containing the extracted lipids to a glass tube suitable for centrifugation. Wash the organic phase with 10 ml of dichloromethane/methanol/water (3:48:47, v:v:v). For phase separation, centrifuge at 6800 x g at room temperature for 10 min and discard the upper phase. Repeat this washing step four times, which ensures the removal of free sugars and salt from the sample. The organic phase is then dried under nitrogen gas.

Dol and Dol-P are purified in two steps using C_{18}- and silica-based chromatography resins. First, equilibrate the C_{18} Sep-Pak cartridge with 5 ml of methanol, followed by 5 ml of chloroform/methanol (2:1, v:v) and 10 ml of methanol/water (98:2, v:v, supplemented with 20 mM phosphoric acid). The dried lipids from the extraction step are dissolved in 400 µl of chloroform/methanol (2:1, v:v) and diluted by adding 10 ml of methanol/water (98:2, v:v) supplemented with 20 mM phosphoric acid. Load the solution onto the C_{18} Sep-Pak cartridge and wash with 20 ml of methanol/water (98:2, v:v, supplemented with 20 mM phosphoric acid). To remove phosphoric acid from the

cartridge, wash again with 10 ml of methanol/water (98:2, v:v) devoid of phosphoric acid. Dol and Dol-P can be eluted with 20 ml of chloroform/methanol (2:1, v:v) and collected in a new glass tube. The eluate should be neutralized by adding 100 µl of 25% ammonium hydroxide.

The second purification step through Silica Sep-Pak cartridges allows the separation of Dol from Dol-P. First, equilibrate the cartridge with 10 ml of chloroform/methanol/water (10:10:3, v:v:v) and 40 ml of chloroform/methanol (2:1, v:v, in 0.5% ammoniumhydroxide). Place a new glass tube to collect the dolichol fraction, which will be in the flowthrough of the chromatography. Apply the neutralized eluate from the previous step onto the Silica Sep-Pak cartridge. Rinse the glass tube of the eluate with 10 ml chloroform/methanol (2:1, v:v, in 0.5% ammoniumhydroxide) and load it onto the Silica cartridge. Repeat the process with10 ml chloroform/methanol (2:1, v:v, in 0.5% ammonium hydroxide). Elute Dol-P into another glass tube by applying 30 ml chloroform/methanol/water (10:10:3, v:v:v) onto the Silica cartridge. Dry the Dol and Dol-P fractions under nitrogen gas at 50 °C.

2.1.3. Anthryldiazomethane labeling of Dol-P

Dol-P labeling is made in two steps. First, Dol-P is dimethylated and then selectively demethylated to produce monomethylated Dol-P (Dol-P-Me). The procedure yields an enhanced fluorescence signal when labeling Dol-P-Me with 9-anthryl derivatives (Yamada et al., 1986). The second step is the derivatization of Dol-P-Me with 9-anthryldiazomethane (ADAM). We use the diazomethane generator system (Sigma-Aldrich) for the dimethylation of Dol-P[1]. It consists of two glass tubes: a small internal tube placed inside a larger outer tube. The dried Dol-P fraction is first dissolved in 1 ml

[1] Diazomethane is very toxic in contact with eye, skin or by inhalation. Diazomethane may explode in contact with sharp edges and when heated beyond 100 °C.

of diethylether and transferred into the large outer glass tube of the diazomethane generator. The glass that contained the Dol-P fraction is washed twice with 1 ml of diethylether, which is added to the outer tube of the diazomethane generator. To produce diazomethane, add 0.367 g Diazald (explosive!) to the inner tube of the diazomethane generator system. Dissolve Diazald by addition of 1 ml of Carbitol. Assemble the two parts of the diazomethane generator system and immerse it into an ice bath. Make sure that the generator system is sealed. Using a syringe, carefully add 1.5 ml of 37% potassium hydroxide dropwise to the inner tube of the diazomethane generator system. Add few drops first and wait until the reaction starts, as seen by formation of gaseous diazomethane in the Diazald solution. In this way, the content of the inner tube is prevented from spilling into the outer tube. Then, carefully add the rest of the potassium hydroxide. Gently mix the reactants and incubate the reaction for 1 h in the ice bath. The diethylether phase containing Dol-P should turn yellow by then, indicating an excess of diazomethane. After disassembly of the diazomethane generator, leftover reactants can be neutralized by addition of 0.15 g of silicic acid to the inner tube. The outer tube should be sealed with parafilm and left for 2 h at room temperature. The Dol-P-Me_2 solution is transferred to a glass extraction tube and dried under nitrogen gas. To selectively demethylate Dol-P-Me_2, dissolve it in 4 ml of *tert*-butylamine and incubate at 70 °C for 14 h. Make sure to seal the extraction tube completely to prevent the loss of *tert*-butylamine. Then, evaporate *tert*-butylamine under nitrogen gas and dissolve Dol-P-Me in 1.5 ml of 0.1 M hydrochloric acid adjusted to pH 3 with sodium hydroxide. Extract Dol-P-Me twice with 3 ml of diethylether and dry the combined extracts under nitrogen gas. Labeling is performed in 1 ml of diethylether saturated with ADAM. Prepare the ADAM solution in a separate glass tube by gradually adding the reddish ADAM powder to 0.7 ml of diethylether until saturation. Take 600 µl of the

yellowish ADAM solution and add it to the dried Dol-P-Me fraction. Incubate the labeling reaction at 4 °C in the dark for 8h. After drying under nitrogen gas, dissolve the reaction products in 2.4 ml of n-hexane. Wash the hexane phase three times with 1.8 ml of acetonitrile. Discard each time the acetonitrile lower phase. Dry the n-hexane phase under nitrogen gas. The ADAM labeled Dol-P-Me fraction can be dissolved in acetonitrile/dichloromethane (3:2, v:v) for HPLC analysis.

2.1.4. HPLC analysis of fluorescently labeled Dol-P

ADAM-derivatized Dol-P are separated by HPLC using an Intertsil ODS-3 column and applying isocratic elution according to Yamada *et al.* (Yamada et al., 1986). The mobile phase consists of acetonitrile/dichloromethan (3:2, v:v) supplemented with 0.01% diethylamine. ADAM fluorescence is detected at 412 nm using an excitatory wavelength of 365 nm.

2.1.5. Negative ion electrospray mass spectrometry of Dol-P

Purified Dol-P can also be directly analyzed by ESI-MS (Haeuptle et al., 2009) without derivatization. The dried Dol-P sample is first dissolved in 90% acetonitrile, 10% n-hexane, containing 0.01% diethylamine, and then vortexed and centrifuged to eliminate any particulate matter. The nano-flow system consists of an injector fitted with a 10 µl fused silica capillary loop, coupled in-line to a nano-flow pump and emitter tip of a nano-electrospray ion source via 20 µm ID fused silica capillary tubing. An aliquot of the sample is loaded into the injector loop and switched in-line to the nano-flow system, introducing the sample directly into the ion source with the dissolution solvent at a flow rate of 400 nl/min. Using a 3200 AB/Sciex QTRAP MS, all mass spectra are acquired

manually in the Enhanced MS scan mode (ion trapping mode), scanning from m/z 1000 to m/z 1700 at a scan rate of 1000 amu/sec and a step size of 0.06 amu. The curtain gas flow is maintained at 10 psi, collision gas pressure at 4 x 10e-5 torr (High), interface heater temperature at 150 °C, declustering potential at -200V, entrance potential at -10V, collision energy at -10V and the ion spray voltage is varied between -2400 and -4500 V, depending on the condition of the emitter needle. Fragment ion spectra of selected Dol-P precursor masses are acquired in the Enhanced Product Ion mode (EPI mode, fragmentation in ion trapping mode) with the collision energy set to -100V and linear scanning starting at m/z 100. Extensive rinsing of the nano-flow system in between sample injections is necessary to avoid cross contamination.

2.2. Dolichol-linked oligosaccharide analysis

Defects of Dol-linked oligosaccharide (DLO) assembly decrease the availability of the mature Dol-PP-$GlcNAc_2Man_9Glc_3$ core for glycosylation of acceptor Asn sites on nascent glycoproteins (Hulsmeier et al., 2007). Such a DLO shortage leads to the non-occupancy of N-glycosylation sites, which impacts on protein folding and trafficking (Helenius and Aebi, 2004). DLOs are conveniently analyzed either by metabolic labeling using [^{3}H]-mannose, or by derivatization with the fluorochrome 2-aminobenzamide (2AB). The extraction, hydrolysis, and purification procedure of DLOs is common to both methods.

2.2.1. Required materials

Chemicals

In addition to the solvents mentioned in section 0, you need the reagents listed below.

- ^{3}H-labeled D-mannose, 5 mCi (Hartmann Analytics, Germany)

- 2-Propanol (Sigma-Aldrich)
- Trizma base (Sigma-Aldrich)
- 2-Aminobenzamide (Sigma-Aldrich)
- Sodium cyanoborohydride $NaBH_3CN$, (Sigma-Aldrich)

Devices and other materials

- Dowex AG 1 x 4, Dowex AG 50 x 8 (Bio-Rad)
- ENVI-C_{18} and ENVI-Carb resin (Supelco)
- Ultrafree-MC centrifugal filter devices (Millipore, Cat. No.: UFC30LH25)
- Supelcosil LC-NH2 column, 5 µm particle size, 250 x 4.6 mm (Supelco)
- Scintillation flow monitor FLO-ONE A-525 (Packard)

2.2.2. [^{3}H]-labeling of DLOs

Approximately 2 x 10^8 are washed three times with PBS to remove Glucose from the medium. Cells are then incubated in DMEM without serum and glucose at 37 °C for 45 min. After this starving step, [^{3}H]-mannose (125 µCi / 10^8 cells) is added to the cells, which are incubated further at 37 °C for 1 h.

2.2.3. Extraction of DLOs

The labeling medium is aspirated and cells are washed quickly with 13 ml of ice-cold PBS. Cells are then fixed by adding 11 ml of ice-cold methanol/0.1 M Tris-HCl (8:3, v:v) and collected by scraping when dealing with adherent cells. The cell suspension is transferred to a 50 ml conical tube and 12 ml of chloroform are added. After vigorous mixing using a vortex, the suspension is centrifuged at 6000 x g for 10 min. The upper methanol phase and the lower chloroform phases are discarded, while the interface

precipitate is kept for further extraction of DLOs. To this end, add 3 ml of chloroform/methanol/water (10:10:3, v:v:v), vortex for 2 min and spin at 6000 x g for 10 min. Transfer the supernatant to a glass extraction tube. Repeat the extraction three times using 2 ml of chloroform/methanol/water (10:10:3, v:v:v). Then dry the extracted DLOs under nitrogen gas.

2.2.4. Hydrolysis and purification of oligosaccharides

Acid hydrolysis is used to release the oligosaccharide from the lipid carrier. For this, incubate the extracted DLOs in 2 ml of 0.1 M hydrochloric acid (in 50% 2-propanol) at 50 °C for 1 h, then neutralize by adding 1 ml of 0.2 M NaOH. To purify oligosaccharides, prepare a disposable chromatography column with 1ml of Dowex AG 1x4 resin, followed by 0.6 ml of Dowex AG 50x8 resin. Equilibrate the column with 20 ml of 30% 2-propanol and apply the 3 ml hydrolyzate, then wash with 3 ml of 30% 2-propanol. Collect the flowthrough (6 ml total) into a new glass tube and evaporate under nitrogen gas. A second column system is used to further purify the DLOs. Prepare a column with 0.2 ml of ENVI-C_{18} resin and 1 ml of ENVI-Carb 120/400 resin. Place a C_{18} Sep-Pak cartridge on top of the prepared column. Wash the system with 5 ml of 100% methanol, 5 ml of 100% acetonitrile and 5 ml of water/acetonitrile/0.1 M ammonium acetate. Finally, equilibrate with 9 ml of 2% acetonitrile/0.1 M ammonium acetate. Resuspend the dried oligosaccharide sample in 1 ml of acetonitrile/0.1 M ammonium acetate and apply it onto the column. Wash the sample vial with 1 ml of acetonitrile/0.1 M ammonium acetate, which is then loaded onto the column. Make sure that the lower ENVI resin column does not run dry during the loading process. Wash the column system with 9 ml of acetonitrile/0.1 M ammonium acetate. To elute DLOs, add 6 ml of water/acetonitrile

(3:1, v:v) and collect the eluate in a new glass tube. Water and acetonitrile are then removed from the pure oligosaccharide sample by evaporation under nitrogen gas.

2.2.5. HPLC analysis of [^{3}H]-labeled oligosaccharides

A Supelco LC-NH2 normal phase column (including a LC-NH2 guard column) and a two solvent system are used for HPLC separation (Zufferey et al., 1995). Prepare acetonitrile/water (7:3, v:v) and acetonitrile/water (1:1) as mobile phases. Dissolve the purified oligosaccharides in 50-100 µl water for injection. The separation is carried out by running a gradient from acetonitrile/water (7:3, v:v) to acetonitrile/water (1:1) over 75 min at a flow rate of 1 ml/min. The [^{3}H]-labeled oligosaccharides are detected using a flow scintillation detector. When the level of [^{3}H]-incorporation is too low for detection by flow scintillation, fractions of the HPLC run can be collected and measured separately in a scintillation counter (Müller et al., 2005). While more time consuming, the latter detection yields a superior signal to noise ratio.

2.2.6. 2-aminobenzamide labeling of DLOs

The labeling of oligosaccharides with 2-aminobenzamide (2AB) is performed according to Bigge *et al.* (Bigge et al., 1995). For 2AB labeling, DLOs are extracted as described under section 0 and oligosaccharides are purified as outlined under section 0. Dried oligosaccharides are resuspended in 200 µl water/acetonitrile (3:1, v:v) and transferred to a 1.5 ml, screw cap Eppendorf tube, where they are dried again. The 2AB labeling reagent is prepared by dissolving 24 mg 2AB in 500 µl acetic acid/DMSO (3:7, v:v) and adding 31 mg sodium cyanoborohydride, thereby achieving a 0.35 M 2AB and 1 M sodium cyanoborohydride solution. Add 20 µl of the labeling reagent to the dried oligosaccharides, mix by vortexing and incubate at 65 °C for 2 h. After cooling, add 380

µl acetonitrile to the sample. Excess labeling reagent is removed using the paper disk clean up procedure (Müller et al., 2005).To this end, punch paper disks from a Whatman 3MM paper using an office hole puncher. Place two paper disks in a centrifugal filter device and wash with 450 µl of water by spinning 30 s at 2000 x g. Wash the filter twice with 450 µl 95% acetonitrile and load the labeled samples. Let the sample pass through the paper disks without spinning the filter device. Wash six times with 450 µl 95% acetonitrile followed by a quick spin at 2000 x g. Elute the labeled oligosaccharides by applying three times 50 µl of water and spinning briefly at 2000 x g.

2.2.7. HPLC analysis of 2AB-labeled oligosaccharides

To separate 2AB-labeled oligosaccharides, we have adapted the procedure of Royle *et al.* (Royle et al., 2002) using a three buffer solvent system (Grubenmann et al., 2004). We use a GlykoSep-N column maintained at 30 °C. Solvent A consists of 80% acetonitrile with 10 mM formic acid (pH 4.4) and solvent B of 40% acetonitrile with 30 mM formic acid (pH 4.4). The 50 mM formic acid stock solution is adjusted to pH 4.4 with ammonium hydroxide. Solvent C is 0.5% formic acid (pH is not adjusted). The gradient is run from 100% solvent A to 100% solvent B over 160 min at a flow rate of 0.4 ml/min. A transition from 100% solvent B to 100% solvent C follows within 2 min at a flow rate of 1 ml/min.

2.3. N-glycosylation Site Occupancy

Most defects of N-glycosylation result in partial occupancy of N-glycosylation sites on proteins. The quantitative analysis of glycosylation site occupancy is not trivial because glycosylated glycosylation sites usually ionize less efficiently than native peptides and it

is not feasible to synthesize adequate standard glycopeptides for quantification purposes. Due to the structural microheterogenity of glycopeptides the relatively low sensitivity in detection is further compromised. Therefore, we developed a strategy to simplify the analysis and quantification of glycosylation site occupancy by deglycosylating the N-linked glycopeptides with N-glycosidase F. As an example, we describe here the analysis of human serum transferrin, which carries two N-glycans at Asn_{413} and Asn_{611}. We showed previously that Asn_{611} glycosylation is most sensitive to cellular stress imposed by congenital disorders of glycosylation or alcohol abuse (Hulsmeier et al., 2007). Serum transferrin is immune-affinity purified from 5 µl serum, proteolytically digested and N-glycans are removed by digestion with N-glycosidase F, which converts the corresponding glycosylated Asn to Asp. N-glycosidase F digestion in buffer constituted with isotopic water ($H_2{}^{18}O$) leading to the incorporation of ^{18}O into the respective Asp, which allows to monitor for potential spontaneous deamination of unoccupied Asn prior to digestion with N-glycosidase F. The inclusion of isotopic labeled standard peptide enables sensitive and accurate quantitation of deglycosylated versus unoccupied glycosylation sites. The generated glycosylation sequon peptides are then analyzed by liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS)[2]. We are using a nanoflow LC (Eksigent) coupled to a hybrid type QTRAP-MS (ABSciex 3200 QTRAP-MS) equipped with a nano-electrospray ionization source. With this instrumentation the triple quadrupole mode MRM-MS can be combined sequentially with product ion scanning in ion trapping mode, or in a MRM signal induced product ion scanning mode. MRM has the advantage that selected fragment transitions are measured resulting in high specificity, sensitivity and reproducible signals for quantitation over a wide range of signal intensities. In our setup, the product ion

[2] MRM is also referred to as single reaction monitoring mass spectrometry (SRM MS) and can be considered synonymous to MRM.

scanning serves as an additional scanning mode to confirm the sequence of the peptide ion selected in the first quadrupole ion filter.

2.3.1. Required devices and materials

- Multiple Affinity Removal LC Column, 4.6 x 50 mm, with proprietary buffer system (MARS LC column “Human 6” or custom made “Human 3” for IgG, transferrin and antitrypsin, Agilent Technologies)
- Iodoacetamide (Sigma Ultra grade)
- Endoproteinase Asp-N (Roche Diagnostics GmbH)
- Trypsin (Roche Diagnostics GmbH, proteomics grade)
- N-Glycosidase F (Roche Diagnostics GmbH)
- Aprotenin (Roche Diagnostics GmbH)
- 20 mM ammonium bicarbonate, dissolved in 97 atom % $H_2{}^{18}O$, Cambridge Isotope Laboratories Inc.)
- Custom synthesized, isotope labeled peptides (≥90% purity, HeavyPeptide Basic kit, Thermo Electron Corp.)
- Multiple reaction monitoring- (MRM-) capable nano-LC-MS system
- C_{18} PepMap 100 trap column (300-µm inner diameter × 5 mm, Dionex)
- C_{18} PepMap100 column (75-µm inner diameter × 15 cm, 3 µm, 100 Å, Dionex)

2.3.2. Purification of transferrin

A sample aliquot containing transferrin corresponding to 5 µl human serum is diluted ten times with Agilent buffer A[3] and loaded onto the MARS column according to the manufacturer instructions. Transferrin will be eluted from the column in one step with 100% Agilent buffer B. The chromatography can be monitored by UV absorption at 280 nm and the transferrin containing peak fraction is collected. The eluate is desalted and concentrated by TCA precipitation. The samples are cooled on ice and adjusted to 12% TCA by adding 0.136 volumes of 100% TCA. The samples are kept on ice for 30 min or ideally at -20°C overnight and centrifuged for 30 min at 14000 x g. The supernatants are discarded and the pellet is washed twice with 300 µl ice-cold (-20 °C) acetone. Centrifuge for 15 min at 4 °C and 14000 x g each time after adding the acetone.

2.3.3. Proteolytic digestion and deglycosylation of glycopeptides

The TCA precipitated protein pellet is dissolved by sonication in 250 µl of 0.57 M Tris-HCl, pH 8.5, 50 mM DTT (1M DTT diluted 20-fold with 0.6 M Tris-HCl, pH 8.5). Proteins are incubated for 5 min at 80°C, cooled to room temperature and cysteine residues are alkylated by adding 63 µl of 1M iodoacetamide (5-fold excess over DTT, freshly prepared). The samples are incubated at room temperature in the dark for 40 min and subsequently desalted by TCA precipitation (see section 2.3.2). The alkylated proteins are re-dissolved in 50 µl of 20 mM ammonium bicarbonate, containing 10% acetonitrile. Then 0.5 µg of endoproteinase Asp-N and 0.5 µg of trypsin are added and the sample is incubated at 37°C for 16 h. Proteolysis is minimized by heating the sample for 5 min at

[3] Buffers A and B are supplied by Agilent together with the MARS column and their composition is proprietary. Therefore, we name these buffers "Agilent buffer A" and "Agilent buffer B", respectively.

80°C, adding 0.5 μmol EDTA and 100 μg aprotinin. Then 8 pmol of each standard peptide is added and the sample is lyophilized overnight. The sample is re-dissolved in 50 μl of 20 mM ammonium bicarbonate in 95 atom % $H_2{}^{18}O$, 1.5 units of N-Glycosidase F (dissolved in 95 atom % $H_2{}^{18}O$) is added and the sample is digested at 37°C for 8 h. The duration of the N-Glycosidase F digestion was empirically optimized and it is recommended to keep these conditions to avoid sample degradation or incomplete N-Glycosidase F digestion. The reaction is stopped by heat inactivation at 80 °C for 5 min. One tenth of the sample is adjusted to 20 μl of 0.1% formic acid in 2% acetonitrile and filtered through a 0.22 μm centrifugal filter device.

2.3.4. Liquid chromatography multiple reaction monitoring mass spectrometry

The sample is loaded on the trap column and the column is washed with 60 μl of 0.1% formic acid, 2% acetonitrile. After washing, the trap column is switched in line to the PepMap column and a binary gradient at 250 nl/min is applied with Buffer A 0.1% formic acid, and buffer B 80% ACN containing 0.1% formic acid. Buffer B is held at 14% for 3 min, increased to 54% over 102 min and to 100% over 8 min, held at 100% for 12 min, and decreased to 14% over 12 min, and the column is re-equilibrated at 14% buffer B for 16 min. The Asn413 peptides elute between 8 and 14 minutes and the Asn611 peptides elute between 22 and 34 minutes retention time. The parameters used for the detection of the transferrin peptides were determined empirically with synthetic peptides and are listed in Table 1.

2.3.5. Calculation of the N-glycosylation site occupancies

The average counts per second (cps) for each MRM transition are calculated by integrating the counts over the time of peak elution with the instrument acquisition software. The molar relative response factor (MRRF) for each peptide sequence is calculated by dividing the cps of the target peptide to the cps of the corresponding standard peptide.

$$MRRF = \frac{cps_{\text{Target peptide}}}{cps_{\text{Standard peptide}}}$$

The percentage of site occupancies is calculated as follows:

$$\%\ at\ Asn_{611} = \frac{MRRF_{DVT}}{MRRF_{DVT} + MRRF_{NVT}} \times 100$$

$$\%\ at\ Asn_{413} = \frac{MRRF_{DK} + MRRF_{DKS}}{MRRF_{DK} + MRRF_{DKS} + MRRF_{NK} + MRRF_{NKS}} \times 100$$

3. O-GLYCOSYLATION

Mucin-type glycosylation is initiated in the Golgi apparatus and is therefore not directly involved in the UPR. However, mucin-type O-glycosylation is involved in the intracellular processing of glycoproteins and contributes to their stability at the cell surface.

3.1. Release of O-glycans by the β-elimination reaction

O-glycans linked to the hydroxyamino acids Ser or Thr can be released by mild alkali treatment. This mild alkali treatment induces the β-elimination reaction, releasing O-linked carbohydrates from the β-carbon of Ser or Thr and leads to the formation of 2-aminopropenoic acid or 2-amino-2-butenocic acid, respectively (Figure 1). A decrease of Ser or Thr after mild alkali treatment can be monitored by amino acid analysis, providing further information about the presence of O-glycosylation and the hydroxyamino acid involved in the O-glycosyl linkage (Hülsmeier et al., 2010; Hülsmeier et al., 2002). The released O-glycans are unstable under alkaline conditions and undergo stepwise degradation reactions termed "peeling reaction". A second β-elimination reaction occurs at the reducing end GalNAc residue, resulting in the formation of a furanosyl compound, the Morgan Elson chromogen (Figure 1). The "peeling reaction" can be minimized by including sodium borohydride ($NaBH_4$) to the reaction mixture, thereby reducing the aldehydic group of GalNAc to the corresponding primary alcohol N-acetylgalactosaminitol (GalNAc-ol). GalNAc-ol is stable under alkaline conditions. Further GalNAc can be isotopically labeled by using either sodium borodeuterite ($NaBD_4$) or sodium borotritiate as reducing agents. A 2:8(mol:mol) mixture of $NaBH_4$ and $NaBD_4$ can be used, which introduces a "fingerprint" isotope distribution into the O-glycans, facilitating the identification of O-glycans in MALDI-MS and alleviating the assignments of fragmentation ion spectra by marking the reducing end C1 carbohydroxy group (Hülsmeier et al., 2002).

3.1.1. Required devices and materials

- Sodium borohydride, sodium borodeuterite (Sigma)
- Sodium hydroxide, purest available grade (Sigma)

- Clean glass rod and glass tube
- Methyl iodide (ReagentPlus, 99.5%, Sigma-Aldrich)
- sodium thiosulphate (Sigma)
- 2,5-dihydroxybenzoic acid (Fluka)

3.1.2. Reductive β-elimination

The glycoprotein sample (1 mg or less) is lyophilized in a 1.5 ml screw cap polypropylene vial and dissolved in 200 µl freshly prepared 0.1 M sodium hydroxide, containing 0.2 M $NaBH_4$ and 0.8 M $NaBD_4$ (prepared by mixing 1M $NaBH_4$ and 1M $NaBD_4$ solutions 2:8 by volume). The sample is incubated at 37°C for 24 h. Then further 100 µl of 0.1 M sodium hydroxide, containing 0.2 M $NaBH_4$ and 0.8 M $NaBD_4$ are added and incubation continues for further 48 h at 37°C. The reaction is stopped by carefully adding acetic acid until gas development ceases. The sample will be acidified to approximately pH 4.

3.1.3. Purification of the β-eliminated O-glycans

A C18 Sep-Pak cartridge is conditioned with 5 ml methanol, 5 ml propanol and equilibrated with 2 times 5 ml 1% acetic acid. A 0.6 ml Dowex AG50W-X12 column is conditioned with 3 times 5 ml 4 M HCl and washed with water until the flow through becomes neutral. The Sep-Pak cartridge is mounted on top of the Dowex column and both columns are equilibrated with 3 times 5 ml of 1% acetic acid. The β-elimination products are passed through the combined columns and the columns are washed twice with 2 ml of 1% acetic acid. The flow through is collected into a glass vial and dried

under vacuum evaporation. Residual boric acid is removed by co-evaporation twice with 250 µl 1% acetic acid in methanol and twice with 250 µl methanol. Residual acetic acid is removed by two evaporations with 50 µl of toluene.

3.1.4. Permethylation

The permethylation derivatization is carried out according to the NaOH method (Ciucanu and Kerel, 1984). The purified O-glycans are dried from 100 µl of 10 mM triethylamine to improve the solubility of negatively charged glycans as triethylamine salts in dimethyl sulfoxide (DMSO). Then, 50 µl DMSO is added and the reaction vial is agitated for 20 min. A 120 mg/ml slurry of NaOH in DMSO is prepared by crushing NaOH pellets with a glass rod in a glass tube. The NaOH will not dissolve completely and precipitate. Mixing of the slurry is required prior application to the derivatization reaction. A 50 µl aliquot of the NaOH/DMSO slurry is added to the reaction vial containing the O-glycans and the vial is shaken for additional 20 min. Then, 10 µl of methyl iodide are added twice, and the vial is agitated for 10 min after each addition, followed by a final addition of 20 µl of methyl iodide and agitation of the vial for further 20 minutes. Permethylated glycans are extracted by adding 250 µl of chloroform and 500 µl of 1 M sodium thiosulphate. The vial is agitated thoroughly and the liquid phases are separated by centrifugation. The aqueous phase is discarded and the chloroform phase, containing the permethylated O-glycans is washed 10 times with 1 ml of water. The chloroform phase is vacuum dried and the permethylated O-glycans are re-dissolved in 50 % acetonitrile for application to MALDI-mass spectrometry (MADLI-MS).

3.1.5. MALDI-MS

The MALDI matrix is prepared by dissolving 10 mg 2,5-dihydroxybenzoic acid (DHB) in 1 ml of 50 % acetonitrile, containing 1 mM sodium chloride (Hülsmeier et al., 2010). Aliquots of the permethylated O-glycans are mixed on the MALDI plate with DHB matrix 1:1 (v:v) and allowed to dry at room temperature. The dried spots are re-crystallized by applying less than 0.1 µl of ethanol. A 10 µl pipette tip is dipped into ethanol and some solvent will be taken up by capillary force. Ethanol outside the tip is evaporated by waving the tip in the air for a few seconds. Then the tip is placed over the dried DHB spot, that the ethanol solvent just touches the MALDI plate surface. The DHB crystals will dissolve quickly and are left to air dry. As a result an even layer of DHB crystals will be formed, permitting sensitive detection of the analytes. The re-crystallization can be repeated, if necessary. Additional DHB matrix can be added, if difficult samples are to be analyzed. MALDI mass spectra are recorded in positive ion mode and glycans are mainly detected as their sodium ion adducts, due the presence of 1 mM sodium chloride in the DHB matrix.

3.2. Release of O-glycans by hydrazinolysis

Releasing O-glycans by hydrazinolysis has the advantage, that the glycans are liberated with a free reducing end saccharide. This allows subsequent labeling with fluorophores like 2AB or anthranilic acid, facilitating high resolution chromatographic separation combined with unparalleled sensitive fluorescence detection (see section 2.2.6-2.2.7, Figure 2). It is important for this reaction to occur under anhydrous conditions and that the sample is free of salt, metal ions, detergent, dyes and stains. The reaction mechanism during hydrazinolysis has not been elucidated so far. However, an initial formation of a

hydrazone derivative with concomitant release of water seems to be likely. After re-N-acetylation of the released glycans, acetohydrazone derivatives are formed and O-glycans in unreduced form can be recovered after passage through cation exchange resin and the addition of Copper-(II)-acetate in mild acid (Patel et al., 1993).

3.2.1. Required devices and materials

- Water and methanol rinsed, oven dried 250 µl glass syringe
- Glass reaction vials
- Lyophilizer
- Pure anhydrous hydrazine (Ludger Ltd, UK)
- Ice cold, saturated sodium bicarbonate solution
- Acetic acid anhydride (Sigma)
- Dowex AG50 [$^{+}$H-form] resin (Bio-Rad)
- Copper-(II)-acetate (Sigma)
- Supelclean™ ENVI-18 resin (Sigma)

3.2.2. Hydrazinolysis

The glycoprotein sample is dialyzed against 0.1% TFA and transferred to a clean glass reaction vial. The sample is lyophilized extensively for one to three days, depending on the amount of protein (up to 5 mg protein). Then, anhydrous hydrazine is added immediately using a glass syringe, pre-rinsed with hydrazine. Hydrazine is added in excess to the sample to give a less than 5 mg protein per ml hydrazine solution. The reaction vessel is capped securely and mixed gently to bring the majority of sample into

solution. The sample is transferred in a heating block at 60 °C and incubated for 5 h. The sample is cooled to room temperature and vacuum dried or lyophilized. Residual hydrazine can be removed from the sample by three times re-drying from 100 µl methanol and finally evaporation from 50 µl toluene. The vial is placed on ice and 100 µl of ice-cold saturated sodium-bicarbonate solution is added, followed by the addition of twice 10 µl acetic acid anhydride[4]. The sample is mixed and incubated at room temperature for 10 min. Then, a second aliquot of acetic acid anhydride is added and the incubation proceeds for further 20 min. The sample is passed through a 3 ml Dowex AG50 [$^{+}$H-form] column, followed by 4 ml of water. The eluate is collected, dried and re-dissolved in 2 ml of 1 mM Copper-(II)-acetate in 1 mM acetic acid. The sample is incubated at room temperature for 1 h and the O-glycans are purified by passage through a column of 2 ml ENVI-18 resin over 1 ml Dowex AG50 [$^{+}$H-form] resin, eluted with water. The O-glycans are now ready for 2AB-derivatisation and HPLC analysis (see sections 2.2.6 and 2.2.7). 2AB-labeled glycan can also be subjected to permethylation for analysis by MALDI-MS. In our hands, permethylation of 2AB-glycans leads to significant higher signal intensities in MALDI-MS compared to permethylation of alkaline borohydride reduced glycans.

ACKNOWLEDGMENTS

This work was supported by the Swiss National Foundation grant 31003A-116039 to TH.

[4] From our experience, re-N-acetylation with sodium bicarbonate can lead to partial O-acetylation reactions. O-acetylation might are not evident in HPLC analyses, but can be detected in MALDI-MS. Here it can serve as an indicator for the presence of saccharide in precursor ion scanning experiments. Saccharides would be detected in MALDI with a characteristic satellite peak increment of 42 Da.

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Table 1: Peptides used for transferrin glycosylation sites Asn_{413} and Asn_{611}. Amino acids containing the isotope label are marked bold: F, +10 Da; L, +7 Da; A, +4 Da. Due to a misscleavage at Lys_{414}, two sets of peptides are required. Two Asn_{413} sequon variants are generated at approximately equal abundance in the reaction mixture. The fragmentation conditions for each peptide were optimized empirically for maximum signal intensities. Dwell time, time per transition; DP, declustering potential; EP, entrance potential; CEP, collision cell entrance potential; CE, collision energy; CXP, collision cell exit potential.

Designation	Sequence	m/z value		Q3 ion
		Standard peptide	Target peptide	
Asn413-NK	CGLVPV**L**AENYNK	749.4	742.4	y_9
Asn413-NKS	CGLVPV**L**AENYNKS	792.9	785.9	y_{10}
Asn413-DK	CGLVPV**LA**ENYDK	755.9	744.9	y_9
Asn413-DKS	CGLVPV**LA**ENYDKS	799.4	788.4	y_{10}
Asn611-NVT	QQQH**L**FGSNVT	640.3	633.3	b_5-NH_3
Asn611-DVT	QQQHL**F**GSDVT	645.3	635.3	b_9

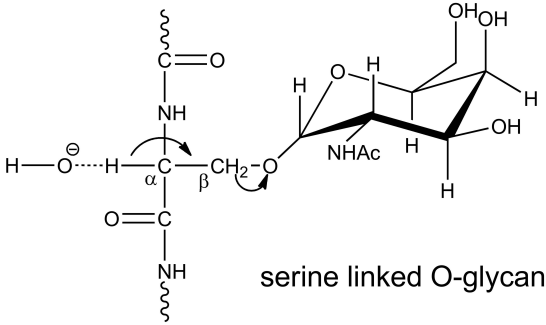
Designation	Dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Asn413-NK	100	40	9.0	40	41	45
Asn413-NKS	100	41	9.5	43	44	48
Asn413-DK	100	41	9.0	40	41	45
Asn413-DKS	100	41	9.5	43	44	48
Asn611-NVT	150	40	9.0	35	37	43
Asn611-DVT	150	40	9.0	35	37	44

FIGURE LEGENDS

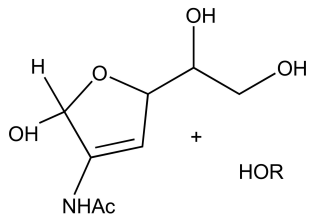
Figure 1: The proposed reaction scheme for the β-elimination reaction is exemplified with a serine linked mucin-type O-glycan. Under alkaline conditions hydroxide ions attack the α-carbon of the amino acid serine, inducing the liberation of the oligosaccharide from the β-carbon. Initially, water and a free reducing end oligosaccharide are generated from the polypeptide and as a result 2-aminopropenoic acid is formed. Inclusion of borohydride to the reaction mixture reduces the oligosaccharide to its corresponding primary alcohol (oligosaccharitol). Without reduction of the oligosaccharide, the glycans would undergo a second β-elimination sequence and the reducing-end GalNac would form the Morgen Elson chromogen. The second β-elimination is accelerated, if the β-carbon is substituted, i.e. participating in a glycosydic linkage.

Figure 2: A fluorescence chromatogram of 2AB-labeled O-linked glycans from bovine submaxillary mucin. The O-glycans were released by hydrazinolysis, derivatized with 2-AB and subjected to normal phase HPLC. The retention times are converted into glucose units (GU) via calibration with a 2AB-labeled dextran hydrosylate in consecutive runs. The calculation of GU facilitates the assignment of glycan structures by comparison with published GU values. These assignments can further be corroborated by digestion with exoglycosidase enzymes. For example, digestion of the sample with sialidase would lead to a reduction of the GlcNAcβ1-3[NeuNAcα2-6]GalNAc-2AB peak and a corresponding increase of the GlcNAcβ1-3GalNAc-2AB peak. The linkage positions are indicated by the orientation of the substituent and are decoded in the displayed pictogram. Anomericity is encoded as a solid line for β-linkage and dotted line for α-linkage, respectively. The

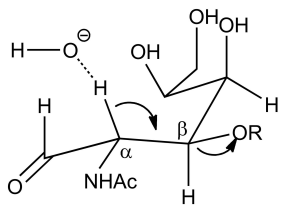
glycan constituents are displayed in different shaped symbols according to the figure legend.

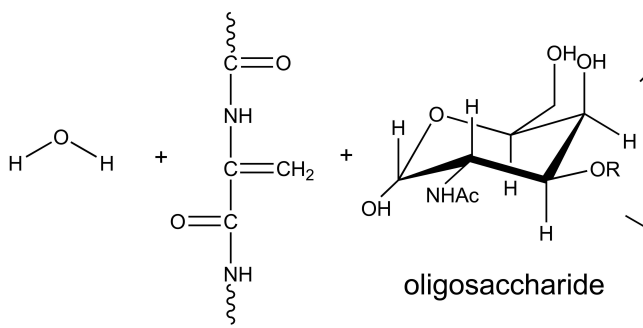


serine linked O-glycan

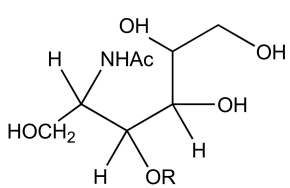


Morgan Elson chromogen



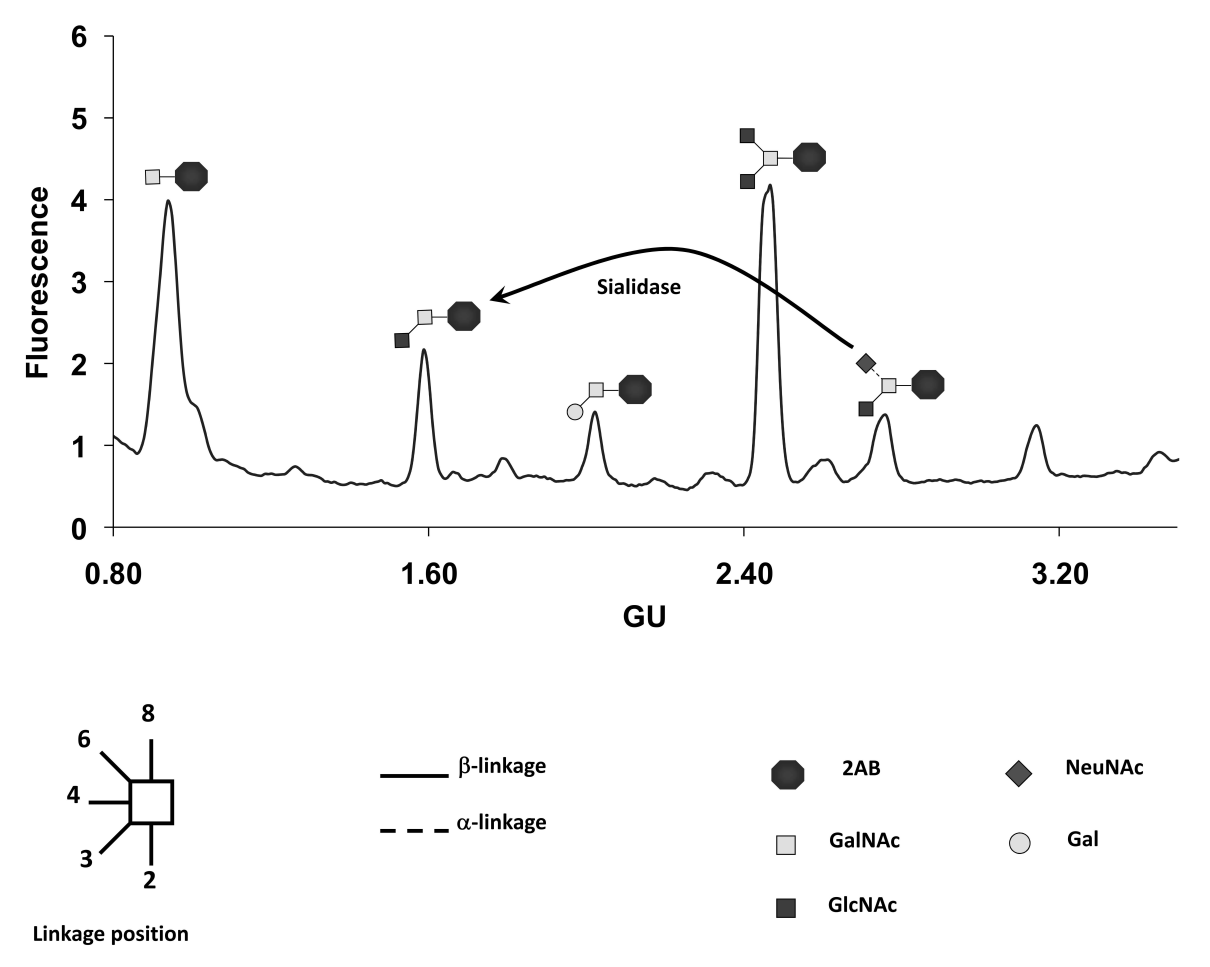


oligosaccharide



oligosaccharitol

2-aminopropenoic acid peptide


Fluorescence
6
5
4
3
2
1
0
Sialidase
0.80
1.60
2.40
3.20
GU
8
6
4
3
2
Linkage position
β-linkage
α-linkage
2AB
NeuNAc
GalNAc
Gal
GlcNAc